Proceedings – 23rd Annual Conference – IEEE/EMBS Oct.25-28, 2001, Istanbul, TURKEY

BIO-FUNCTIONALIZED NEUROCHIPS

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Abstract-**Arrays of gold microelectrodes have been generated on glass chips. Various adhesion molecules have then been covalently bound to the surface. Micropatterns of adhesion molecules were generated using photolithographic techniques. Dissociated neurons from chicken dorsal root ganglia adhere selectively to the adhesion molecules and form networks. It could be demonstrated that a single neuron can be stimulated by an adjacent microelectrode. The neural outgrowth was much improved on the specific adhesion proteins axonin-1 and NgCAM, compared to surfaces treated with aminosilane. Furthermore, the distance of cell membrane to surface was at a minimum on these specific neural adhesion proteins. These results show that the quality of neuron cultures on chips can be improved if specific neural adhesion proteins are used.**

Keywords **- Neuron, adhesion proteins, photolithography, patterning, neural networks, functionalization**

I. INTRODUCTION

Various types of microelectrode arrays (MEA) have been produced using photolithographic techniques. Such MEAs are suitable for stimulating and recording neural activity in slices of nerve tissues or of dissociated neurons in cultures. Two main factors determine the quality of MEA chips: the sensitivity of the microelectrodes for detecting and stimulating neuronal signals and the biocompatibility of the surface.

It is known that positively charged groups present on the surface promote cell adhesion. However, coatings with poly-lysine may not be long-term stable and covalently immobilized molecules are preferred. In order to guide neurite outgrowth towards electrodes we have developed micropatterning techniques. Especially, two recombinant proteins of the specific neural adhesion proteins axonin-1 and Ng-CAM have been designed and produced. These proteins are specific for neurons and expected to form close cell-material contacts. Furthermore, the neural outgrowth can be directed towards electrodes by protein micropatterns with a width down to 1 µm generated by photolithographic methods. The production and characterization of such biofunctionalized neurochips is presented.

II. METHODOLOGY

1) *Adhesion molecules*: Recombinant proteins from the two membrane proteins axonin-1 and NgCAM have been produced using genetic engineering methods as described [1]. The recombinant proteins have a C-terminal Cys, which allows a direct coupling to gold surfaces. Alternatively, a covalent immobilization on glass or oxides can be achieved using silanes and heterobifunctional crosslinkers. By this method the peptide RGDC and the proteins Cys-axonin-1 and Cys-NgCAM have been immobilized.

2) *Chip production:* Microelectrode arrays have been fabricated according to [2]: A 30 nm thin gold film was evaporated on the photolithographically structured resist, which was removed by a lift-off process. The remaining gold lines were insulated with a silicon oxide layer. Optionally a polyimide layer was added with openings of 20 x 100 µm at the position of the microelectrodes, to serve as a topological barrier for neuron cells.

3) *Cell cultures:* Neurons were isolated from chicken dorsal root ganglia and cultured in MEM [1]. The cells were vital for several days.

4) *Fluorescence interference:* The cell-surface distance was determined using the fluorescence interference contrast method [1]. Briefly, the neural adhesion molecules were covalently immobilized on silicon oxide chips and the neurons added. The *mean* membrane-substrate distance was determined by measuring the fluorescence intensity contrast on oxide steps of different heights. From these measurements the distance can be calculated.

5) *Biopatterning:* Standard positive photoresist techniques were adapted to generate micropatterns of covalently immobilized proteins on glass [3]. Both lift-off and plasma etching techniques were used to transfer the patterns into a layer of covalently bound protein. The functionality of the protein was assessed by immunostaining.

6) *Electrophysiology:* The chips were tested in eletrophysiological experiments. A current pulse was applied to a microelectrode close to a neuron cell and the evoked signal was recorded by a patch clamp electrode.

III. RESULTS

A. Neurochip fabrication

A section of the whole neurochip, which has a dimension of 10 x 10 mm, is shown in Fig.1. In the upper part squares of

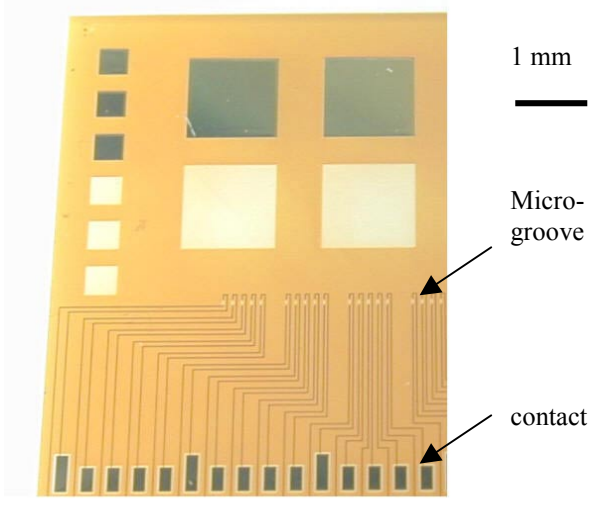

Fig. 1.
Section of the neurochip.

Report Documentation Page

Report Date	Report Type	Dates Covered (from... to)
25 Oct 2002	N/A	-

Title and Subtitle Bio-Functionalized Neurochips	**Contract Number**
	Grant Number
	Program Element Number
Author(s)	**Project Number**
	Task Number
	Work Unit Number
Performing Organization Name(s) and Address(es) Paul Scherrer Institut Laboratory for Micro- and Nanotechnology Villigen, Switzerland	**Performing Organization Report Number**
Sponsoring/Monitoring Agency Name(s) and Address(es) US Army Research, Development & Standardization Group (UK) PSC 802 Box 15 FPO AE 09499-1500	**Sponsor/Monitor's Acronym(s)**
	Sponsor/Monitor's Report Number(s)

Distribution/Availability Statement
Approved for public release, distribution unlimited

Supplementary Notes
Papers from 23rd Annual International Conference of the IEEE Engineering in Medicine and Biology Society, October 25-28, 2001, held in Istanbul, Turkey. See also ADM001351 for entire conference on cd-rom., The original document contains color images.

Abstract

Subject Terms

Report Classification unclassified	**Classification of this page** unclassified
Classification of Abstract unclassified	**Limitation of Abstract** UU

Number of Pages
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gold (black) and of silicon oxide (white) are visible. The microgrooves appear in the middle of the picture; each channel is addressed by two microelectrodes. The contact pads are clearly visible.

Fig. 2 shows an opening in the 15 µm thick polyimide resist layer. The two gold electrodes ending in the microgroove are blank, but otherwise insulated by silicon oxide and additionally by the polyimide layer. This material is biocompatible and its topological structure should prevent migration of neurons. However, neurons attach to the polyimide and the chosen microgroove dimensions turned out to be too small: neurons, which have been positioned into the groove by a micromanipulator, escape from it within one day. By modifying the top of the polyimide with cell repellent molecules cell attachment could be prevented. Furthermore, larger microgrooves will probably be more suitable to prevent cell migration.

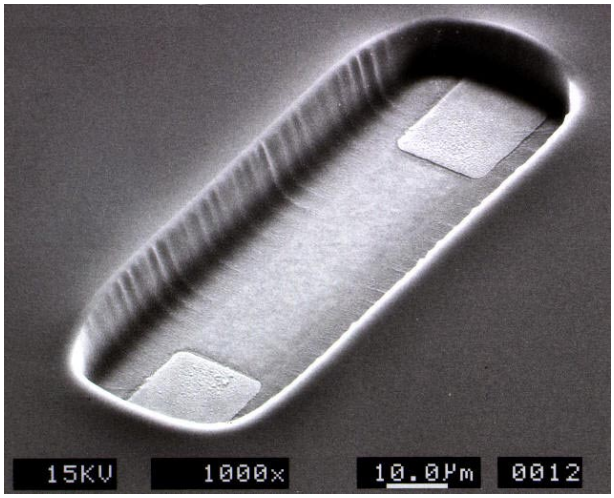

Fig. 2.
SEM image of a microgroove.

TABLE I
NEURONS CULTURED ON VARIOUS SUBSTRATES

Substrate	Mean neurite length[a] (µm)	Membrane / substrate mean distance[b] (nm)
Cys-axonin	80 ± 30	37 ± 10
APTES	40 ± 10	39 ± 3
RGDC	124 ± 60	39 ± 4
Cys-NgCAM	231 ± 86	47 ± 8
Polylysine	n.d.	54 ± 9
Laminin	n.d.	91 ± 4
	[a] n > 30	[b] 7 < n < 14

It has also to be mentioned that neural outgrowth (neurites) can only be observed for 20 % of the adhered neurons when cultured on APTES, whereas on laminin and on NgCAM 80% of the cells have neurites (see Fig. 3). On RGDC and axonin-1 neurite outgrowth was found for about 50% of the inspected neurons. This result shows that neurons sensitively react to the surface chemistry.

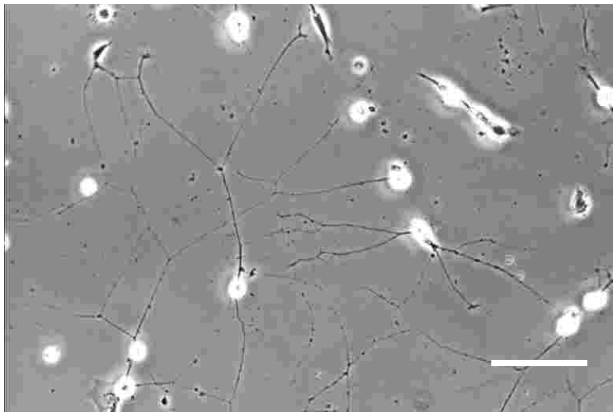

Fig. 3.
Neurons on NgCAM-functionalized glass chips. Note the long neurites (scale bar 100 µm).

B. Bio-Functionalization

Several cell adhesion molecules are known and frequently used for neuroscience experiments. We present here two novel adhesion proteins, which are specific for neurons, axonin-1 and Ng-CAM. In the development of the nerve system they play an important role for the guidance of the neural growth cone [4]. Thus, we can also expect specific reactions, when they are used for *in vitro* experiments. Since the recombinant proteins Cys-axonin-1 and Cys-NgCAM are immobilized in the correct orientation, their full functionality on a surface is retained. Table I summarizes our findings. By microscopic inspection of neurons cultured on glass chips, which have been modified with the mentioned molecules, the mean neurite lengths could be assessed. The neurites are six-times longer on NgCAM and three-times longer on axonin-1 than on the aminosilane APTES used as a control. This is a clear indication that the adhesion mechanism is different on the specific adhesion molecules.

This conclusion is further supported by measurements of the cell membrane-surface distance (see Table I). When cells are cultured on laminin, the mean distance was about 91 nm. On APTES, RGD and axonin-1 a distance of about 40 nm was determined, which seems to be a lower limit. From structural consideration a larger distance is expected, when NgCAM is involved (see Fig. 4). In this case a tetrameric complex is formed between two cells; when only axonin-1 is present on the cell membrane, a smaller complex of two axonin-1 molecules, a homodimer, will be formed. The expected larger distance for NgCAM could experimentally be confirmed. It has to be kept in mind that *mean* distances are given and that the cell membrane at the contact points may be closer to the surface. In summary, the use of specific adhesion proteins for a functionalization of surfaces is attractive for future experiments in neurosciences.

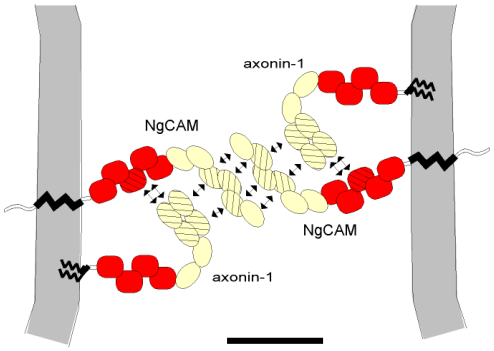

Fig. 4.
Scheme of cell-cell recognition by adhesion proteins
(scale bar about 10 nm).

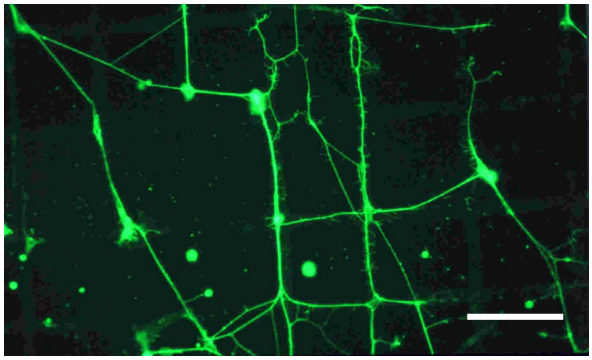

Fig. 6.
Neurons on a pattern of immobilized axonin-1(scale bar 100 µm).

C. *Patterns of adhesion molecules*

Neurons adhere to lines of at least 5 µm width of adhesion molecules immobilized on the chip surface. In this way neurite outgrowth of the neuron cells can be guided to the microelectrodes. Our results demonstrate the feasibility of patterning methods. Lines of RGDC can be produced on large areas by a photolithographic lift-off method: (1) The resist on a wafer is patterned by photolithography, (2) the molecules are immobilized, and (3) the resist is removed. Neurons adhere to the resulting RGDC-peptide lines and extend neurites along the parallel lines (phase contrast micrograph, Fig. 5). More delicate is the production of axonin-1 patterns. We could recently show [3] that a pattern of functional proteins can be produced, if the protein is protected by embedding in a sucrose film during photolithographic structuring. Neurites align to the axonin-1 patterns and can be visualized by immunostaining with fluorescein-anti-axonin-1 (Fig. 6).

The lift-off method can also be used for a local immobilization of proteins on 3D-structures. As a model protein rabbit-IgG is covalently immobilized at the bottom of the microgroove on an area, which is restricted by the photoresist. The immobilized protein is then visualized using an anit-rIgG-rhodamine conjugate and appears grey in Fig. 7. The polyimide is strongly autofluorescent at λ =552 nm and appears white.

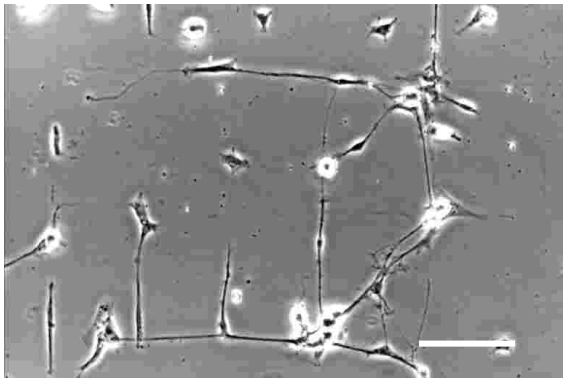

Fig. 5.
Neurons on a pattern of immobilized RGD (scale bar 100 µm).

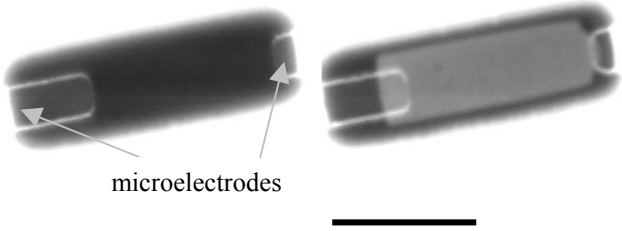

Fig. 7.
Localized immobilization of proteins into 3D-microstructures.
In this fluorescence micrograph a microgoove before (left) and after (right) IgG immobilization and immunostainig is shown (scale bar 50 µm).

D. *Electrophysiological experiments*

The neurochip was integrated into an adaptor for electrophysiological experiments (Fig. 8). In Fig. 9 a picture of the experimental setup is given, as used for the patch clamp experiment.

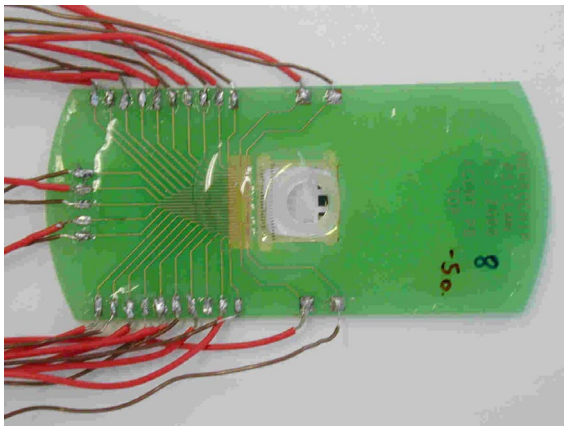

Fig. 8.
A neurochip (below the white ring in the middle) has been bonded into a printed circuit board for stimulation/recording experiments.

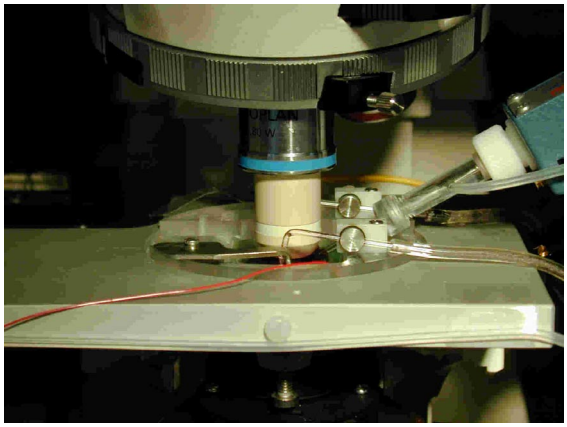

Fig. 9.
Picture of the experimental setup used to test the neurochips:
The objective of the microscope and the micromanipulator for positioning the patch clamp pipette can clearly be recognized.

A cell adjacent to a microelectrode was stimulated and the evoked signal was recorded intracellularly (Fig. 10). In this preliminary experiments RGDC-treated chips and 4-7 days old neuron cultures were used. It could be demonstrated that the gold microelectrodes are useful for neuron stimulation. However, further improvements are required to *record* extracellularly electrophysiological signals from stimulated cells.

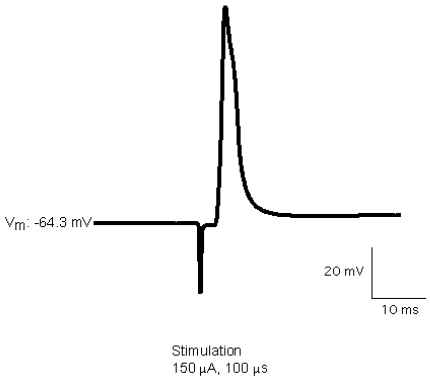

Fig. 10.
Action potential recorded intracellularly using a patch clamp micropipette after extracellular stimulation.

IV. Discussion

The presented results show that a MEA of gold electrodes in combination with a polyimide layer is suitable for neural network cultures. However, the chips were not yet long-term stable and the insulation layer has to be improved. Furthermore, the dimensions of the microgrooves have to be enlarged in order to achieve a cell adhesion at the bottom of the microgroove and simultaneously to prevent cell migration. Biopatterns of adhesion molecules are helpful to align neural outgrowths. Whether they also can improve the quality of the recorded electrophysiological signals is still unknown. However, it could be shown that these novel adhesion molecules promote neurite outgrowth. In addition, these and probably further specific neural adhesion molecules may also be involved in synaptogenesis. This assumption makes them very attractive for experiments in the future.

V. Conclusion

We presented new procedures for the production of neurochips to establish neural networks. Although many methods have to be improved further, some conclusion can be drawn. First, gold electrode arrays may be especially attractive, because a direct coupling of thiol-compounds can be achieved. The stability of such surface modifications in physiological fluids is questionable, due to a putative degradation within days. Nevertheless they can be essential for the formation of neural networks at the beginning. Second, specific adhesion molecules improve some biological reactions of cells on surfaces. It is still unknown if the observed benefit on the neural outgrowth is relevant for signal detection. However, it can be claimed that polylysine or amino-terminated molecules are not physiological molecules for cell adhesion. Various reactions of neurons will be induced by their adhesion on surfaces, like for instance a rearrangement of the cytoskeleton. Such biological reactions will depend on the surface chemistry and may have an impact on further cell functions and finally also influence the quality of neural networks. Future research will elucidate the significance of a specific bio-functionalization.

Acknowledgment

This work has been supported by the priority program “Biotechnology” of the Swiss National Science Foundation, Module Neuroinformatics. The authors thank Peter Sonderegger, University of Zürich, Lukas Leder, Novartis AG and Sandra Ruch for their substantial support in cell culturing and recombinant protein production. We also thank K. Ballmer, PSI, for his support in microscopy. The technical support of Ulf Gensser for mask design, of Theresa Mezzacasa, Dieter Bächle, Fredy Glaus, Bianca Haas and Brigitte Ketterer for clean room processes at PSI is acknowledged.

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